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Tetracycline fluorescence in uremic and primary hyperparathyroid bone

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Tetracycline fluorescence in uremic and primary hyperparathyroid bone. Twenty-five patients with end-stage renal disease, nine of whom were receiving pharmacologic doses of vitamin D, and seventeen patients with primary hyperparathyroidism underwent bone biopsy following a three-day course of tetracycline administration. The mean width of the fluorescent tetracycline bands were significantly greater in the bones of patients with uremia than in those with primary hyperparathyroidism. This difference was due to wide labels present in the patients with uremia who had not been treated with vitamin D, as no differences existed in mean label widths of patients with uremia who had received this compound and the patients with primary hyperparathyroidism. Comparison of the maximum label widths distinguished not only primary hyperparathyroid patients from those with uremia, but uremic patients who had recieved vitamin D from those who had not been so treated. Quantitative microscopy of standard, nonfluorescent histologic features failed to make this latter distinction. These data are consistent with the presence of a wide zone of instantaneously fluorescing material in uremic bone following tetracycline administration, which does not relate to bone apposition occurring during antibiotic administration. This phenomenon probably represents a delay in mineral maturation which is normalized by vitamin D. Furthermore, it is apparent that the use of a continuously administered (single) tetracycline label will result in an overestimation of bone formation rates, particularly in osteomalacic states.

Fluorescence de la tétracycline dans l'os au cours de l'hyperparathyroïdisme urémique ou primaire. Vingt-cinq malades atteints d'insuffisance rénale terminale, dont neuf recevaient des doses pharmacologiques de vitamine D, et dix-sept malades atteints d'hyperparathyroïdie primaire, ont subi une biopsie osseuse après l'administration de tétracycline pendant trois jours. La largeur moyenne des bandes de fluorescence de la tétracycline est significativement plus grande dans l'os de malades urémiques que dans celui de malades atteints d'hyperparathyroïdie primaire. Cette différence est due aux marques larges qui existent chez les malades urémiques qui n'ont pas été traités par la vitamine D. En effet, il n'y a pas de différence entre la largeur des marquages chez les urémiques traités par la vitamine D et les malades atteints d'hyperparathyroïdisme primaire. La comparaison des largeurs maximales ne différencie pas seulement l'hyperparathyroïdisme primaire de l'urémie, mais encore les urémies traitées par la vitamine D de celles qui ne l'ont pas été. La microscopie quantitative des caractères habituels ne comprenant pas la fluorescence, ne peut pas faire une telle différentiation. Ces résultats concordent avec la présence dans l'os, au cours de l'urémie, d'une large zone de matériel immédiatement fluorescent après l'administration de tétracycline qui ne traduit pas une apposition osseuse qui surviendrait au cours de l'administration de l'antibiotique. Ce phénomène représente probablement un retard de la maturation minérale, retard qui est corrigé par la vitamine D. Il apparaît, de plus, que l'emploi d'un marquage par la tétracycline administrée de façon continue aboutit à une surestimation de la vitesse de formation de l'os, surtout au cours des ostéomalacies.

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Since the discovery of tetracycline-induced fluorescence in bone [1], the study of human skeletal tissue has had the unique advantage of a nontoxic, tissue time-marker which may be used to obtain meaningful kinetic data by morphologic means [2]. The fundamental assumption underlying many such histomorphometric studies is that the quantity of fluorescent tissue produced during administration of the antibiotic reflects net bone mineralization which has occurred during that period [3–8].

As a result of a previous study performed in this laboratory [9], we became interested in the degree to which the total quantity of tetracycline fluorescence in lamellar bone may not represent time-related bone synthesis. We have therefore examined the widths of fluorescent labels following tetracycline administered over a short period of time to a group of patients with end-stage renal disease or primary hyperparathyroidism. The significant differences in label width between these groups may be related to the status of mineralization.

Methods

The first population studied consisted of 13 male and 12 female, randomly selected patients with chronic uremia (mean age, 39.2 ± 14.9 [SD] yr), who had been on maintenance hemodialysis an average of 35 months. All patients were dialyzed three times a week against a dialysate calcium of 6.5 mg/dl. Fol-

lowing selection of these patients, their charts were reviewed for history of vitamin D therapy, and their current skeletal x-ray films were quantitated according to the method of Debnam et al [10].

The second group studied was comprised of 17 consecutively encountered patients with primary hyperparathyroidism. The group included 11 males and 6 females (mean age, 55.7 ± 11.3 [SD] yr).

Each patient received one gram of oxytetracycline per day in four divided doses for three days, immediately prior to transiliac biopsy with a trocar (I.D., 0.5 cm). On the day of biopsy, blood was obtained for measurement of total calcium, phosphorus, alkaline phosphatase, and immunoreactive parathyroid hormone (iPTH) [11]. Bone biopsy was performed on the uremic patients under local anesthesia, and during parathyroidectomy on those with primary hyperparathyroidism.

Each bone biopsy specimen, which consisted of both cortices and intervening trabeculae, was fixed in neutral buffered formalin for 24 hr, embedded in methylmethacrylate, and cut in 10-μm sections on a sledge microtome (Jung, model K). Unstained sections were examined in a fluorescent microscope (Leitz Ortholux) by a microscopist with no knowledge of the source of the slide. Employing a filar micrometer, we measured the width of the fluorescent label at 25 points where present on straight portions of trabeculae, as determined by random interception of the cross hairs of the micrometer, and expressed as the mean label width of each patient, and the mean label width of the group. The maximum of each 25 measurements was identified in each patient as the maximum label width; and the mean of these values, as the average maximum label width of the group.

To compare the relative specificity of the tetracycline-based measurements to those routinely evaluated in nondecalcified thin sections of bone, a 5.0-μm section of each specimen was stained by a modification of the Goldner technique and histometrically quantitated using an integrating eyepiece (Zeiss II) as previously described [9]. The following nonfluorescent histologic parameters were evaluated: *1)* percentage of relative osteoid volume (ROV), or the percentage of trabecular bone matrix which is unmineralized; *2)* percentage of total osteoid surface (TOS), or the percentage of trabecular surface covered by unmineralized bone matrix; *3)* percentage of osteoblastic osteoid surface (OOS), or the percentage of trabecular surface covered by osteoid lined by plump osteoblasts; *4)* number of osteoclasts per mm^2 of cancellous space (OCL); and *5)* percentage of surface fibrosis (FIB), or the percentage of trabecular surface in apposition to marrow fibrosis.

The differences in measured parameters between groups were evaluated by Student's t test. In addition, correlations between the magnitudes of each nonfluorescent parameter and the tetracycline-based variables were examined. Any such relationships were evaluated within the groups of biopsies of patients with primary hyperparathyroidism or renal failure, as well as those of uremic patients who had been treated or untreated with vitamin D. They were also evaluated among the biopsies of all 42 patients considered as a single group.

Results

Clinical. Nine of the 25 patients with chronic renal failure had been taking vitamin D for at least three months. The dosage administered to seven patients was 50,000 IU of vitamin D_2 daily, while two patients received an identical dose tri-weekly and bi-weekly, respectively. There were no differences in the ages nor the duration of dialytic therapy of the vitamin D and non-vitamin D-treated patients.

There were distinctions between the circulating biochemical parameters of these two groups, however (Table 1). The mean total calcium was higher ($P < 0.05$) and the phosphorus lower ($P < 0.05$) in those patients receiving ergocalciferol. Furthermore, while circulating iPTH was increased in all patients with chronic renal failure, it was more than four-fold greater in those who had not received vitamin D_2 ($P < 0.025$). On the other hand, the skeletal x-rays of the vitamin D-treated patients exhibited more severe renal osteodystrophy ($P < 0.025$).

Sixteen of the 17 patients with primary hyperparathyroidism were hypercalcemic (Table 1). Eight patients were hypophosphatemic, and seven had elevated levels of circulating alkaline phosphatase. Circulating iPTH levels were determined in all but one patient and was increased in each, including the individual with a normal serum calcium concentration.

No patient with primary hyperparathyroidism complained of symptoms referable to the skeletal system. Seven patients, however, had radiographic abnormalities. Five had generalized skeletal demineralization, while two had evidence of subperiosteal resorption. One of the two latter patients also had a lesion consistent in appearance with osteitis fibrosa cystica.

Histometric. The mean label width of the group of patients with uremia was greater than twice that of those with primary hyperparathyroidism (Table 2). Whereas only six percent, or one of 17 primary hyperparathyroid patients had mean label widths

Table 1. Mean values ± SD of circulating biochemical parameters (at time of bone biopsy) of patient groups studied[a]

	Normal range	All patients with CRF	CRF patients treated with vit. D	CRF patients not treated with vit. D	Patients with PHP
Calcium, *mg/dl*	9 to 10.5	9.9 ± 1.4	10.7 ± 1.5	9.5 ± 1.0	12.1 ± 0.8
Phosphorus, *mg/dl*	2.5 ± 5.0	5.0 ± 1.5	4.1 ± 1.2	5.5 ± 1.5	2.6 ± 0.6
Alkaline phosphatase, *IU*	<100	84 ± 54	83 ± 72	84 ± 45	107 ± 42
iPTH, *μlEq/ml*	<10	354 ± 359	115 ± 131	491 ± 378	39 ± 41

[a] Abbreviations used are: CRF, chronic renal failure; PHP, primary hyperparathyroidism; iPTH, immunoreactive parathyroid hormone.

Table 2. Mean values ± SD of histometrically measured bone biopsy parameters of patient groups studied[a]

Patient group	Mean label width	Maximum label width	Relative osteoid volume, %	Total osteoid surface, %	Osteoblastic osteoid surface, %	Osteoclasts/mm² of cancellous space	Surface fibrosis, %
PHP	5.94 ± 2.35	15.5 ± 4.81	8.56 ± 4.35	44.6 ± 17.6	8.93 ± 7.47	1.06 ± 0.77	2.68 ± 6.86
vs. all CRF	12.4 ± 10.9[b]	44.2 ± 33.6[d]	11.5 ± 5.88	71.8 ± 15.0[e]	5.39 ± 5.79	1.06 ± 1.02	10.2 ± 15.3
PHP	5.94 ± 2.35	15.5 ± 4.81	8.56 ± 4.35	44.6 ± 17.6	8.93 ± 7.47	1.06 ± 0.77	2.68 ± 6.86
vs. CRF (vit. D-treated)	7.30 ± 4.5	21.5 ± 15.0	10.8 ± 6.95	69.1 ± 18.05[d]	4.38 ± 7.74	0.88 ± 1.12	6.45 ± 16.3
PHP	5.94 ± 2.35	15.5 ± 4.81	8.56 ± 4.35	44.6 ± 17.6	8.93 ± 7.47	1.06 ± 0.77	2.68 ± 6.86
vs. CRF (no vit. D therapy)	15.5 ± 12.5[d]	57.0 ± 34.7[e]	11.9 ± 5.39	73.3 ± 13.5[e]	5.96 ± 4.55	1.16 ± 0.98	12.3 ± 14.8
CRF (vit. D-treated)	7.30 ± 4.5	21.5 ± 15.0	10.8 ± 6.95	69.1 ± 18.05	4.38 ± 7.74	0.88 ± 1.12	6.45 ± 16.3
vs. CRF (no vit. D therapy)	15.3 ± 12.5	57.0 ± 34.7[c]	11.9 ± 5.39	73.3 ± 13.5	5.96 ± 4.55	1.16 ± 0.98	12.3 ± 14.8

[a] Abbreviations used are PHP, primary hyperparathyroidism; CRF, chronic renal failure.
[b] $P < 0.025$.
[c] $P < 0.010$.
[d] $P < 0.005$.
[e] $P < 0.001$.

greater than 10 μm, this was evident in 44% of uremic patients (Fig. 1). The increased mean label width of the uremic patients, relative to those with primary hyperparathyroidism, was due entirely to those who had not been treated with vitamin D. No significant difference in mean label width existed between the group of renal patients who had been treated with vitamin D and individuals with primary hyperparathyroidism (Table 2). In addition, 22.2% of uremic patients treated with this compound had a mean label width greater than 10 μm, while this value was exceded in the biopsies of 56.2% of untreated patients with renal failure (Fig. 2).

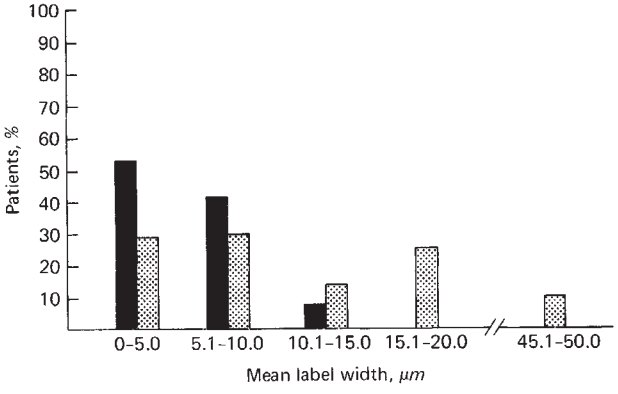

Fig. 1. *Distribution of mean label widths of patients with primary hyperparathyroidism (solid) and renal failure (dotted).*

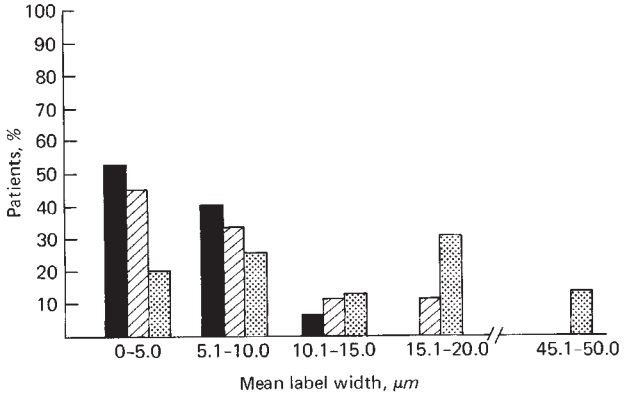

Fig. 2. *Distribution of mean label widths of patients with primary hyperparathyroidism (solid), renal failure treated with vitamin D (diagonal), and renal failure untreated with vitamin D (dotted).*

The average maximum label widths of the two large groups were even more strikingly different than the mean label widths. The average maximum label width of all patients with uremia was approximately three-fold greater than that of the patients with primary hyperparathyroidism (Table 2). Similar to the mean label width, the relatively large magnitude of the uremic average maximum label width was due exclusively to measurements made in those untreated with vitamin D. Not only was there a significant difference between the average maximum label widths of the primary hyperparathyroid group and the untreated uremic group, while no such distinction existed between the treated uremic and primary hyperparathyroid patients, but the average maximum label width of the untreated uremic patients was significantly greater than that of those patients who had received vitamin D. Notable differences in the distribution of the maximum label widths within the two major groups were also present. Whereas no patients with primary hyperparathyroidism had a maximum label width greater than 30 μm, this was present in 60% of uremics (Fig. 3), and 75.1% of those who had not received vitamin D (Fig. 4).

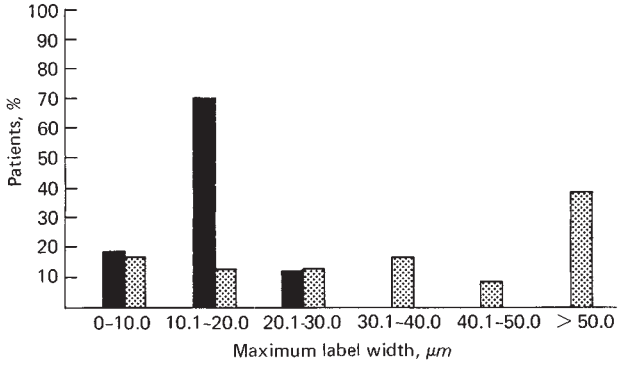

Fig. 3. *Distribution of maximum label widths of patients with primary hyperparathyroidism (solid) and renal failure (dotted).*

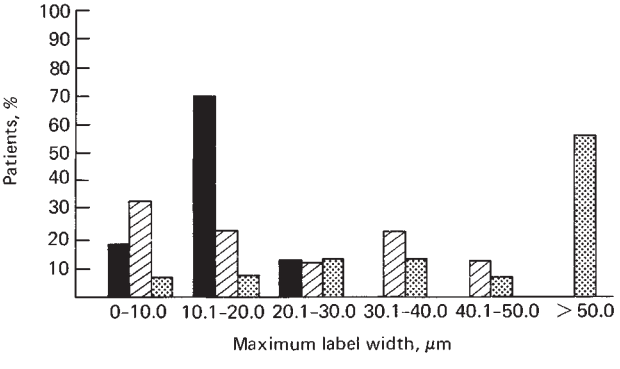

Fig. 4. *Distribution of maximum label widths of patients with primary hyperparathyroidism (solid) and renal failure treated with vitamin D (diagonal) and untreated with vitamin D (dotted).*

Nonfluorescent histologic features did not define the two major groups as clearly as did the tetracycline-based parameters. Only the percentage of trabecular surface covered by osteoid (TOS) was significantly different in the uremic and primary hyperparathyroid patients (Table 2). In addition, whereas average maximum label widths were significantly different in the two uremic subgroups, no nonfluorescent histologic feature distinguished those patients with renal failure who had received vitamin D from those who had not been treated with this compound. Moreover, there were no correlations between nonfluorescent and fluorescent morphometric parameters in any group of patients.

Discussion

Nondecalcified histologic sections of bone are indispensable for the morphologic evaluation of renal osteodystrophy. Of major importance is that by the use of these sections, one is able to distinguish mineralized from nonmineralized bone matrix. Consequently, it has become appreciated that the morphologic diagnosis of osteomalacia necessitates histologic demonstration of an excess quantity of osteoid (hyperosteoidosis).

While investigators assume excessive osteoid is pathognomonic of osteomalacia [12], hyperosteoidosis may theoretically represent an increased rate of bone matrix deposition in face of a normal mineralization rate. Such a condition is believed by Sherrard et al to exist in uremic patients whose bone biopsies exhibit a predominance of osteitis fibrosa [13].

The distinction between hyperosteoidosis due to a mineralization defect and that secondary to an increased rate of bone collagen synthesis may be achieved by use of tetracyclines as fluorescent markers of bone mineralization [14]. These antibiotics are characteristically deposited in newly synthesized bone [1, 15–17]. Consequently, the tetracyclines have become invaluable, *in vivo* markers of new bone formation [3–8]. As has been demonstrated by Taylor and Frost [18], however, a finite quantity of skeletal tissue exists at the interface of osteoid and mineralized bone, which binds the antibiotic instantaneously, and therefore does not represent bone mineralization which has occurred during its administration. Consequently, the total quantity of fluorescent bone present following a period of tetracycline administration reflects the sum of bone which has been mineralized during that period, and the instantaneously fluorescing zone.

This study demonstrates that the widths of single fluorescent labels administered over a relatively

short period of time are distinct in uremic as compared to primary hyperparathyroid bone. Furthermore, while one nonfluorescent histometric parameter (TOS) is significantly different in the biopsies of patients with primary hyperparathyroidism, as compared to those with chronic renal failure, no correlations exist between any fluorescent and nonfluorescent parameter. Consequently, the tetracycline label width may reflect a phenomenon which cannot be appreciated by examining nonfluorescent histologic sections.

As the fluorescent band represents the sum of bone which has been mineralized during the period of tetracycline labeling and the instantaneously fluorescing zone, the magnitude of the label width in uremia can only be related to *1)* an accelerated rate of bone mineralization, *2)* prolongation of the circulating half-life of tetracycline, and/or *3)* a wide instantaneously fluorescing zone. A decreased rate of bone mineralization exists in chronic renal failure [19, 20], however, and it is therefore unlikely that accelerated bone formation has occurred in our uremic patients. In addition, while the circulating half-lives of the tetracyclines are prolonged in uremia [21], the degree of renal insufficiency was identical in both our vitamin D and nonvitamin D-treated patients, despite differences in label width. Furthermore, abnormally wide labels occur in hypophosphatemic osteomalacia [9], and in vitamin D deficient rats [22] in the absence of renal failure. Therefore, the wide fluorescent labels encountered in our uremic patients are most likely representative of the instantaneously fluorescing zone. As such, the use of a continuously administered (single) tetracycline marker to measure skeletal formation will overestimate the quantity of matrix mineralized, particularly in states with a wide instantaneously fluorescing zone. This problem may be circumvented by administering time-spaced (double) fluorescent markers, as proposed by Frost [23]. This technique rests on the assumption that skeletal mineralization is in a steady state and that the width of the first instantaneously fluorescing zone equals that of the second. As such, tissue mineralized from the middle of administration of the first label to the middle of the second is reflected by the quantity of bone encompassed by the midpoints of the fluorescent bands.

In lamellar bone, tetracycline is largely chelated at the interface of osteoid and mineralized bone [24], variously called the calcification or mineralization front. In this regard, we have not observed in these or other histologic sections of bone labeled with the antibiotic, the diffuse, low-level fluorescence reported in deep interstitial lamellae by Urist and Ibsen [15] and Harris, Jackson, and Jowsey [16]. It is of interest that the latter investigators [16] observed this phenomenon only following administration of doses of tetracycline far in excess of those used in this study. Furthermore, they describe diffuse, interstitial, low-level fluorescence exclusively in areas where some degree of increased calcium 45 uptake is autoradiographically apparent. This finding indicates that while matrix synthesis may have ceased in these foci, the rate of mineral deposition is greater than in surrounding deep bone.

The thickness of our histologic sections may also relate to our failure to observe diffuse, low-level interstitial fluorescence. Urist and Ibsen [15] and Harris et al [16] studied sections five to ten times thicker than those prepared in our laboratory, which would be expected to result in greater sensitivity to fluorescence. Furthermore, as osteocytes are capable of mineralization activity [25], perilacunar, tetracycline fluorescence [16] outside of the plane of focus may contribute to the diffuse interstitial phenomenon observed in thick sections.

Accumulated evidence suggests the mineral phase at the calcification front is probably amorphous calcium phosphate [26], the form in which bone mineral is most likely deposited [27]. With time, amorphous calcium phosphate is transformed into crystalline hydroxyapatite, which characterizes the deeper portions of the skeleton [27]. As tetracycline is incapable of binding to normal deep bone in quantities sufficient to produce the degree of fluorescence present in the biopsies of our patients, it is reasonable to assume that the instantaneously fluorescing zone reflects amorphous calcium phosphate existing at the time of tetracycline administration. Therefore, it is likely that the quantity of amorphous calcium phosphate at the mineralization front of patients with uremia is greater than in those with primary hyperparathyroidism. This is consistent with the demonstration of a delay in mineral maturation in experimental uremia by Russell, Termine, and Avioli [28].

The failure of nonfluorescent histometric parameters to reflect fluorescent label width probably relates to the inability of currently employed histologic stains to distinguish immature from mature bone mineral. For example, woven bone which fluoresces diffusely following tetracycline administration [29], and therefore probably contains increased quantities of amorphous calcium phosphate, appears tinctorially similar to lamellar bone when stained by the Goldner technique. In this regard, the data presented in this study may call for redefinition of the histologic manifestations of osteomalacia. This defect has been characterized by an excess of osteoid seams, few of which assume a tetracycline label [30]. As has been demonstrated in this laboratory [9] as well as in

others [13], states of deficient mineralization, including chronic renal failure [13], exist in which a normal percentage of such seams assume a tetracycline label, albeit wide and irregular. This abnormality suggests that there are at least two kinetic defects of mineralization associated with osteomalacia. Deficient rates of deposition of amorphous calcium phosphate may be reflected by an absence of fluorescence at the mineralizing front, while wide fluorescing fronts may represent a delay in the maturation of amorphous calcium phosphate to its crystalline phase. The relative normalization of the fluorescent label width occurring in uremic patients treated with vitamin D suggests this compound may accelerate the rate of mineral maturation in renal osteodystrophy, as has been shown experimentally by Russell et al [31]. As more radiographic evidence of renal osteodystrophy existed in the vitamin D-treated patients than in those of patients not receiving ergocalciferol, despite lower levels of circulating iPTH and phosphorus, fluorescent histologic parameters are earlier harbingers of skeletal improvement than x-ray examination. Furthermore, despite longitudinal studies demonstrating amelioration of renal osteodystrophy, as evaluated by nonfluorescent histology following treatment with analogs of vitamin D [32, 33], our data suggest the sensitivity of these nontetracycline-based parameters, as representative of vitamin D-induced effect on the uremic skeleton, may not be as great as fluorescent label width. Consequently, histologic evaluation of the state of bone mineralization in uremia may require measurement of fluorescent label width as well as mineralization rate and osteoid volume.

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